SPATIAL DISTRIBUTION AND LOCALIZATION OF ColE1-Ap PLASMID DEOXYRIBONUCLEIC ACID ISOLATED FROM ESCHERICHIA COLI AFTER CHLORAMPHENICOL AMPLIFICATION

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

BIOLOGY

Gosier, Kim Y. B.S., Spelman College, 1977
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Spatial Distribution and Localization of ColE1-Ap Plasmid Deoxyribonucleic Acid Isolated from Escherichia coli after Chloramphenicol Amplification

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Examination of the intracellular localization of pulse-labeled ColE1-Ap molecules after chloramphenicol (Cm) exposure revealed a high level (42%) of membrane-bound plasmid DNA on cesium chloride (CsCl)-sucrose double density equilibrium gradients. Appreciable amounts of ColE1-Ap DNA were released from the membrane-plasmid DNA complexes by vortexing prior to centrifugation. The isopycnic step neutral sucrose gradient technique was employed to elucidate the specific bacterial cell membrane layer involved in the complexing of amplified ColE1-Ap DNA. The cytoplasmic (CM) or inner (IM) membrane layer predominated the association of 95% of the CM-incubated plasmid molecules to the cell envelope after centrifugation. The proteins involved in the apparent association of Cm-incubated ColE1-Ap molecules were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Analysis suggested that the high

molecular weight (MW) proteins isolated from exponential and Cm amplified freely sedimenting forms of ColE1-Ap were also visualized in gel tracks which contained samples of IM and Cm isolates. Data, therefore, support the attachment of amplified ColE1-Ap DNA to cytoplasmic membrane proteins found complexed to CM proteins located at the zones of adhesion in the bacterial cell membrane. Further, we favor the premise that the amplified ColE1-Ap molecules aggregate at these zones as a "bunch of grapes on a vine" for the purpose of replication and segregation.

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Special thanks to my parents for their confidence in me. Super thanks Mama....you said that it was possible. Thank God, for you made it possible.

CHAPTER I

INTRODUCTION

R-factors are autonomously replicating, infectious bacterial plasmids. These extrachromosomal elements carry resistance to antibiotics which may be conferred upon their host.

Cascading studies have been conducted relative to the maintenance of bacterial plasmids. Several studies imply that the replicational events of the host chromosomal deoxyribonucleic acid (DNA) occur via a membrane vehicle. More specifically, it has been reported that the origin of the chromosomal DNA associated with a 31,000 molecular weight (MW) protein of the bacterial cell outer membrane. Evidence suggesting that bacterial plasmid DNA is also attached to a membrane vehicle for the purpose of replication and/or segregation has been questionable.

Numerous investigations have suggested that several plasmid DNAs co-sedimented with the host bacterial chromosome in neutral sucrose gradients, when a highly folded chromosomal structure was maintained. This highly looped structure was stabilized by ribonuclease (RNase) and protein.

The size of the plasmid and its specific DNA polymerase contribute to the degree of plasmid complexing with the bacterial exponential nucleoid (chromosome and membrane). For example, plasmids

dependent upon DNA polymerase III are 80-90% associated, while those using DNA polymerase I are weakly associated (approximately 10%).

The R-factor, Colicin-El-Ampicilian (ColEl-Ap), has been observed in actively growing cultures of Escherichia coli. Analysis of exponential cultures utilizing neutral sucrose gradients revealed that 90% of these plasmid DNA molecules were released as freely-sedimenting forms. Exposure of the culture to the antibiotic chloramphenicol (Cm) influenced a complete reversal in this spatial distribution, such that 90% of the in vivo ColEl-Ap plasmid DNA molecules were found associated and only 10% remained uncomplexed.

The cesium chloride-sucrose double density equilibrium gradient is another useful tool which has been employed for further analysis of the bacterial nucleoid and complexed plasmid DNA. This gradient allows one to separate unassociated or free DNA from membrane associated DNA from free membrane on the basis of density differences. Experimentation suggests that during exponential conditions the majority (91%) of DNA molecules band as free DNA in the CsCl-sucrose gradient. On the contrary, after treatment with Cm more than 50% of the plasmid DNA band in the membrane associated DNA region. Labeling conditions assured that only radioactive ColEl-Ap DNA was apparent after Cm incubation.

In light of these observations, further experimentation was devised. The major objectives were: (i) To further analyze the specific membrane layer (inner membrane or outer membrane) involved in the anchorage of ColEl-Ap molecules utilizing the isopycnic step neutral sucrose gradient; and (ii) to possibly identify a specific membrane protein predominating in the apparent adhesive quality of the membrane via gel electrophoresis.

CHAPTER II

REVIEW OF LITERATURE

Jacob et al. (1963) postulated that the chromosome of Escherichia coli attaches to the bacterial cell membrane to stimulate the initiation of DNA replication. Further, growth of the cell envelope between two such attachment sites provides the structural basis for chromosomal segregation in the bacterium. Presently, there has not been any direct evidence to suggest that bacterial plasmids attach to bacterial cell membranes during replication or segregation or both.

Studies by Kline and Miller (1975) have shown that a variety of plasmid DNAs in E. coli associated with their host bacterial chromosome in neutral sucrose gradients, provided the chromosome was maintained in a highly-looped folded configuration (Worcel and Burgi, 1972). It was demonstrated by Stonington and Pettijohn (1971) that this folded structure of the bacterial genome is stabilized by RNA and protein. Further, treatment of this highly looped bacterial nucleoid (chromosome and membrane complex) with RNase resulted in an unfolded chromosomal structure (Drlica and Worcel, 1975; Pettijohn and Hecht, 1973). Archibold et al. (1978) have revealed that 80% of the R6K plasmid DNA co-sedimented with the bacterial nucleoid under conditions for maximum preservation of the folded state. Similarly, Kline et al. (1978) have indicated that 90-100% of F

plasmid DNA in E. coli. is associated with the folded bacterial nucleoid. On the other hand, Kline et al. (1976) provided evidence which suggested that in exponentially growing cells of E. coli, the majority (90%) of nonreplicating molecules of ColE1 plasmid DNAs are found unassociated with the folded chromosome.

Kline and Miller (1975) postulated that plasmids may interact directly with the folded chromosome at sites involving RNA molecules, without direct insertion. This postulation was based on the physiochemical structure of the chromosome, as proposed by Pettijohn and Hecht (1973), as well as observations that plasmid DNA co-sedimented with the 1800S folded chromosomal structure, presumably without associated membrane (Kline and Miller, 1975; Woldarczyk and Kline, 1976). Evidence that membrane is involved in plasmid-chromosomal complexes in vivo has been limited and indirect (Korn and Thomas, 1971). However, Sheehy et al. (1977) were able to release the plasmids, R6K and F, from chromosomal associations by treating the chromosome with RNase.

The size of a plasmid, as well as the kind of DNA polymerase used to replicate the plasmid, contributes to the degree of complexing. Miller et al. (1978) indicated that ColE1 (4.2 Mdal) is 10% associated, and uses predominantly DNA polymerase I for replication (Kingsbury and Helinski, 1970). Conversely, R6K (26 Mdal) and F (60 Mdal) are up to 90% associated and use DNA polymerase III predominantly (Thompson and Broda, 1973). It is apparent that many plasmids that associate at high frequencies are frequently under stringent control of replication via DNA polymerase III. Stringent

plasmids replicate during the period of chromosomal replication and relaxed plasmids do not have an obligate coupling to chromosomal replication.

The rate of chromosomal DNA replication in bacteria is controlled by the frequency of initiation (Maaloe and Kjeldgaard, 1966). The initiation process requires ribonucleic acid (RNA) (Messer, 1972) and protein synthesis (Lark et al., 1963). RNA synthesis is required to provide messengers for the initiator proteins or as a primer for DNA synthesis (Blair et al., 1972; Brutlag et al., 1971). Protein synthesis provides "initiator proteins" which interact at the origin of replication to start DNA synthesis (Lark, 1969). Several studies have shown that many requirements for replication are shared by the host chromosome and plasmid (Arai and Clowes, 1975; Bazaral and Helinski, 1970). However, Womble and Rownd (1979) have demonstrated increases in R-plasmid DNA (NRI) in E. coli. after inhibition of protein and/or RNA synthesis. More specifically, ColE1 DNA replicates at an amplified rate after the inhibition of protein synthesis by chloramphenicol (Cm) (Clewell, 1972). Additionally, ColE1 DNA accumulates ribonucleotides during the incubation of E. coli in Cm. These RNA molecules have been proposed to serve as primers during the initiation of ColE1 replication. Blair et al. (1972) indicated that RNA-primer-rich ColE1 was sensitive to digestion by RNase, unless the culture was exposed to rifampicin, an inhibitor of the initiation of RNA synthesis (Sipple and Hartman, 1968). It was also noted by Blair et al. (1972) that incubation of cultures in Cm inhibited the removal of RNA "primers" from circular ColE1-DNA. Further, chlo-

ramphenicol and rifampin cause condensation of the nucleoid (bacterial chromosome and membrane) to the center of the cell and a concomitant release of chromosomal DNA from the membrane (Wolf-Watz and Norqvist, 1979).

The cell envelope of gram negative bacteria is an extremely complex structure consisting of three morphologically and biochemically distinct layers: the cytoplasmic membrane, the peptidoglycan layer, and the outer membrane (Wolf-Watz and Norqvist, 1979). A full genetic complement is received by each daughter cell after the growth and division of the bacterial cell.

A strict correlation must exist between envelope growth, cell division, DNA replication and DNA segregation. Jacob et al. (1963), as previously mentioned, suggested that the attachment of the bacterial chromosomal DNA to its bacterial cell membrane was involved in the regulation of DNA synthesis and segregation into daughter cells. Nicolaidis and Holland (1978), using the step neutral sucrose density equilibrium gradient to separate inner membrane (IM) or cytoplasmic membrane (CM) from outer membrane (CM), suggested that chromosomal DNA (approximately 1%, equivalent to a maximum of 3 binding sites) was possibly bound to regions of adhesion between cytoplasmic and outer membrane. These zones sedimented on the gradient with a density indistinguishable from that of outer membrane. However, their data also suggested that cultures synchronized for DNA replication showed a two-eightfold enrichment for chromosomal origin DNA, specifically in the outer membrane fractions. While these isolates of CM fractions did not appear to contain replication fork

DNA, small amounts of replication fork DNA were observed in the inner membrane complexes. Olsen et al. (1974) and Portalier and Worcel (1975) have isolated E. coli chromosomal DNA attached to a high density envelope component, which they suggested to be junction regions between inner and outer membrane. Gomez-Eichelmann and Bastarrachea (1975) reported the association of an origin-like DNA with isolated inner membrane fractions, and suggested that binding of DNA to outer membrane occurred during membrane preparation and was non-specific. Wolf-Watz and Norqvist (1979) contradicted this observation when they demonstrated via gel electrophoresis that approximately 0.40% of the total chromosomal DNA was specifically bound to outer membrane via a 31,000 MW protein. The stability of the inner and outer membrane fractions has been tested and data suggested that CM complexes were quite stable against shearing as well as Sarkosyl and DNase digestion, while IM DNA complexes in contrast were quite labile and readily disassociated to release free DNA (Nicolaidis and Holland, 1978). ColE1-Ap plasmid DNA has been isolated with cell membrane fragments on cesium chloride-sucrose double density equilibrium gradients (Gosier, 1979). The specific membrane fragment (outer membrane and/or inner membrane) involved in association with a specific plasmid has not been clearly defined.

The nucleotide sequence of the origin of ColE1 DNA replication and of a small ColE1-type plasmid have been determined. A model for the possible role of the relaxation complex in ColE1 DNA replication and/or conjugal transfer has been presented (Sparks and Helinski, 1979). A major feature of the model is that the relaxation complex

proteins may help promote association of plasmid DNA with a replicator and/or conjugal transfer site on the bacterial membrane. Evidence has been presented with CsCl-sucrose gradients suggesting that membrane-like structures attach to ColE1 DNA and mini-ColE1 DNA in a region that includes both the site of the origin/terminus of DNA replication, and the site of single strand cleavage by the relaxation complex (Sparks and Helinski, 1979).

Many proteins, integral and peripheral, have been associated with bacterial cell membranes. Specifically, the cell membrane proteins include those proteins associated with the outer membrane, the cytoplasmic membrane and the periplasmic space. The bacterial cell outer membrane proteins have been extensively reviewed by DiRienzo et al. (1978). The outer membrane proteins of wild type E. coli K-12 contain at least three classes of major proteins: Matrix proteins, ompA protein, and lipoprotein. Matrix proteins are characterized by their tight, but noncovalent, association with the peptidoglycan. Results indicated that the function of the matrix proteins is to form passive diffusion pores and because of this property, they are also called "porins" (Nakae, 1976). One matrix protein "porin" is protein Ia from E. coli (MW approximately 36,500), consisting of a single polypeptide of 336 amino acid residues.

The second type of CM protein is the ompA protein. This protein has a molecular weight of 30,000. The existence of this protein is an important requirement of F^+ mediated conjugation (Manning and Achtman, 1979). Outer membrane proteins detach only if heated

above 60 C in a strong detergent, or if ionic bonds are weakened by a high salt concentration.

Lipoproteins are the most abundant proteins in the bacterial cell, relative to numbers of molecules (Inouye, 1979). These proteins play an important role in maintaining the integrity of the outer membrane structure. Braun and Rehn (1969) first reported the existence of lipoprotein covalently bound to peptidoglycan. This protein has a molecular weight of approximately 7,200.

Minor proteins also exist in bacterial outer cell membranes, however, they may become major proteins when their production is fully induced. Approximately 10-20 minor proteins are present in the outer membrane. Many minor proteins, in addition to the major outer membrane proteins, have been identified as receptors for phages and colicins. On the other hand, many of them have no known receptor functions, but are now known to have vital roles in the growth of the cell, such as the uptake of nutritional substrates through the outer membrane (DiRenzo et al. 1978).

There are large numbers of proteins in the inner membrane. They include proteins involved in transport, electron transport, and biosynthesis of cell wall constituents and DNA replication.

The periplasmic space sequesters potentially destructive enzymes between inner and outer membrane. The periplasm also contains specific binding proteins functional in membrane transport and chemotaxis (Davis et al., 1980).

The outer membrane has been associated, as previously indicated, with the replication and segregation of chromosomal DNA. A 31,000

molecular weight protein has been isolated in this association. To date, no evidence has directly illustrated that plasmid DNA is maintained in the bacterial cell via the cytoplasmic cell membrane or the outer cell membrane, or both. There is also no evidence suggesting the predominance of a specific protein in observed plasmid DNA-membrane associations. There is, however, evidence which suggests that the outer cell membrane contains receptors for the colicins of colicinogenic plasmid DNA molecules.

CHAPTER III

MATERIALS AND METHODS

Media and Culture Conditions

The synthetic medium used was a minimal salts solution (Curtiss, 1965) supplemented with 0.5% casamino acids, 0.5% glucose, 2 mg/ml thiaminehydrochloride, and ampicillin (40 µg/ml). Routinely, overnight static cultures in supplemented media were inoculated the next morning at 1/10 their volume into supplemented synthetic media and shaken on a water bath shaker (Lab-line) maintained at 37 c. After 30 min equilibration, cultures were labeled during exponential growth either with 1µCi[^{14} C]-dThd, 10µCi[^{3} H] - dThd, or 1 Ci/ml [^{14} C] - glycerol (Moravek Biochemicals, CI, California). The former two radioactive labels were supplemented with adenosine (200 µg/ml, Sigma Chemical Co., St. Louis, MO) to aid incorporation. Cell density was monitored spectrophotometrically at 620 nm using a Buchler Spectronic-20. Exponential cultures reached an optical density (O.D.) of 0.3 (3X10^{8} cells/ml) in all experiments before further handling.

Analysis of Plasmid DNA on "CLOS Gradients"

When the appropriate cell density was reached, exponential cultures were immediately harvested by centrifugation (5C) at 10,000

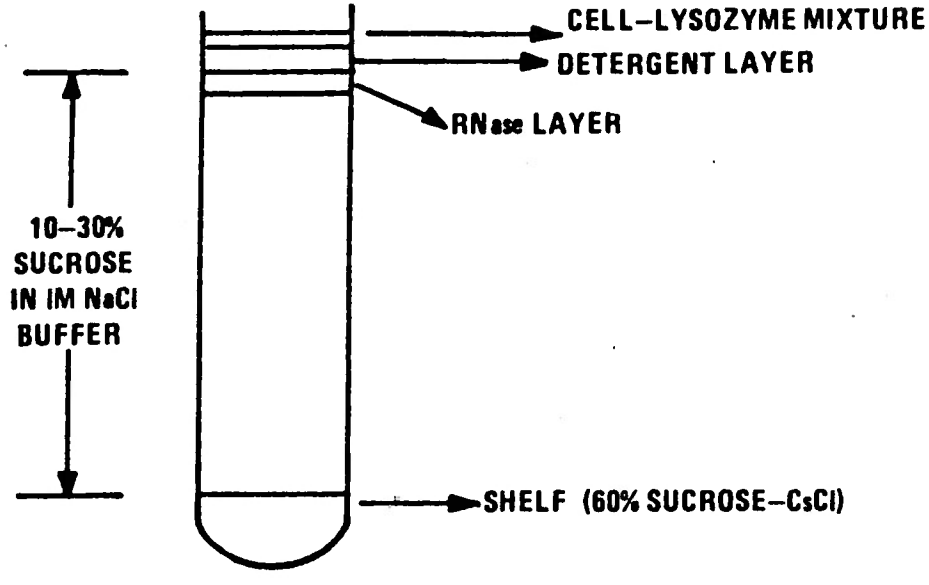
rpm for 10 min, then resuspended at 1/100 the volume in cold buffer consisting of 0.02 M KCN, 0.5 M NaCl, 0.01 M ethylenediaminetetra-acetic acid (EDTA), and 0.01 M Tris (hydroxymethyl) amino methane (Tris-HCL), made up in 5% sucrose at ph 8.0. A 0.4 ml portion of the concentrated cell suspension was mixed with 0.1 ml of lysozyme (30 mg/ml stock; Sigma Chemical Co.) freshly prepared in EDTA-Tris buffer with the appropriate NaCl concentration but without sucrose, and incubated for 15 min. A 0.2 ml aliquot of this mixture was immediately mixed with 0.25 ml of 10% Brij-58 detergent made up in the 5% sucrose-EDTA-Tris buffer and allowed to incubate at 0-4 C for 30 min. A 0.45 aliquot of this mixture was immediately layered onto a 4.5 ml 10 to 30% neutral sucrose gradient maintained at 5 C. The body of the gradient contained 0.25 ml of a bottom shelf of saturated CSCl in 60% sucrose.

Presented in Fig. 1 is a diagram of the Cleared Lysate on Sucrose (CLOS) gradient. After layering of the cell suspension, the gradients were centrifuged under conditions listed in the Figure legends in Beckman L5-50 or L5-65 ultracentrifuges. The gradients were collected on strips or in trays and handled as indicated by Sheehy et al. (1978).

Chloramphenicol (Cm) Amplification and Pulse-Labeling

Exponential cultures were pre-labeled with 10 μCi/ml of [^{14}C]-glycerol to pre-label membrane material. When the O.D. reached 0.3, 200 μg/ml Cm was added and aeration continued for 2 hr. The culture was pulse-labeled with 30 μCi/ml of [^{3}H]-dThd plus adenosine

Fig. 1. A profile showing the components of a 10-30% neutral sucrose gradient.


CELL–LYSOZYME MIXTURE
DETERGENT LAYER
RNase LAYER
10–30%
SUCROSE
IN IM NaCl
BUFFER
SHELF (60% SUCROSE–CsCl)

for 1 min and appropriate aliquots were removed, poured over frozen crushed supplemented synthetic media containing 0.02 M KCN to rapidly inhibit metabolic activity. Cultures were harvested by centrifugation (10,000 rpm, 10 min, 5 C) and handled as indicated previously. The chase experiments were omitted in most cases; however, several cultures were analyzed over longer incubation periods, i.e. up to 5 hr.

Cesium Chloride-Sucrose Double Density Gradient Analysis

Pooled samples of CLOS gradients were dialyzed for 45 min in a phosphate buffer (0.05 M sodium phosphate, 0.005 M EDTA-pH 7.4) and mixed on a Vortex mixer for 1 min at the highest speed setting. Mixing times were divided into (2) 30 s intervals and samples were placed on ice for 5 min between intervals.

The double linear gradients of CsCl and sucrose (10-20% w/w) were formed in 11.0 ml centrifuge tubes using a peristaltic pump (Orion Instruments, Inc.). The initial solutions were prepared as indicated by Sueoka and Hammers (1974). Solid CsCl (0.084 g/ml) was added to 10% sucrose (w/w) prepared in a buffer containing 10 mM Tris, 30 mM EDTA, and 50 mM NaCl, pH 8.0 (TEN buffer). For the 20% solution, 0.84 g/ml of solid CsCl was added to 20% sucrose (w/w) prepared in TEN-buffer. The gradients contained a bottom shelf (0.5 ml) of saturated CsCl in 60% sucrose. Samples (up to 0.5 ml) of cell suspensions were layered on top of the gradients and centrifuged in a SW41 Beckman rotor at 36,000 rpm for 18 hr at 5 C. Collection of gradients was as described by Sheehy and Novick (1975).

Separation of Cytoplasmic and Outer Membrane Fractions by Isopycnic Density Gradient Centrifugation

Pooled samples from CLOS nucleoid regions as well as samples from plasmid regions were dialyzed in phosphate buffer (previously described) and layered on a 30-50% sucrose gradient.

These density gradients were prepared, as described by Osborn et al. (1972), in 11.0 ml centrifuge tubes (using gentle step-wise layering techniques) by layering 1.8 ml each of 50, 45, 40, 35, and 30% sucrose solutions (w/w) over a cushion (0.5 ml) of 55% sucrose. One milliliter of the sample suspension was layered on top of the gradient, and centrifugation was carried out at 38,000 rpm for 16 hr at 0-4 C. Gradients were fractionated by puncturing the bottom of the tube and collecting drops.

Polyacrylamide Gel Electrophoresis

Gels containing a 3% stacking gel and a 10% separating gel were prepared from a stock solution of 30% (w/w) acrylamide and 0.8% (w/w) N,N'-bis-methlyene acrylamide (Lammeli and Favre, 1976). The final concentrations in the separation slab gel were as follows: 0.375 M Tris-HCL (pH 8.8) and 0.1% sodium dodecyl sulfate. The gels were polymerized by the addition of 0.025% by volume of tetra-methylethylenediamine (TEMED) and ammonium persulfate. The electrode buffer (pH 8.3) contained 0.025M Tris-HCL, 0.192 M glycine, and 0.1% SDS. The samples (1.0 ml) contained 0.0625 M Tris-HCL (pH 6.8), SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue

as the dye. Proteins were disassociated by immersing the samples in a 60 C water bath for 1.5 min. Electrophoresis was carried out with a current of 10 mA until the bromophenol blue marker reached the bottom of the gel (about 20 hr).

The gels were fixed and simultaneously stained for 1 hr at 37 C with a 0.25% Commassie brilliant blue solution made up freshly in 45% methanol and 10% acetic acid. The gels were diffusion destained by repeated washing in a solution containing 10% methanol and 10% acetic acid.

Samples subjected to polyacrylamide gel electrophoresis were pooled from freely sedimenting plasmid regions of CLOS gradients, inner and outer membrane regions of the isopycnic step neutral sucrose gradients, and the nucleoid regions of CLOS gradients. Both the nucleoid samples and the plasmid DNA samples were dialyzed (as previously indicated) prior to electrophoresis.

Standard protein markers were electrophoresed along with the other samples to provide an index of approximate MW determinations. The marker proteins utilized was: Bovine albumin (66,000 MW), egg albumin (45,000 MW), B-lactoglobulin (36,500 MW), pepsin (24,000 MW), trypsinogen (24,000 MW) and lysozyme (14,300 MW).

Samples isolated from RS86 generally contained low µg of protein per ml, according to the methods of Lowry et al. (1951). It was, therefore, necessary to overload the gels in order to visualize protein banding patterns.

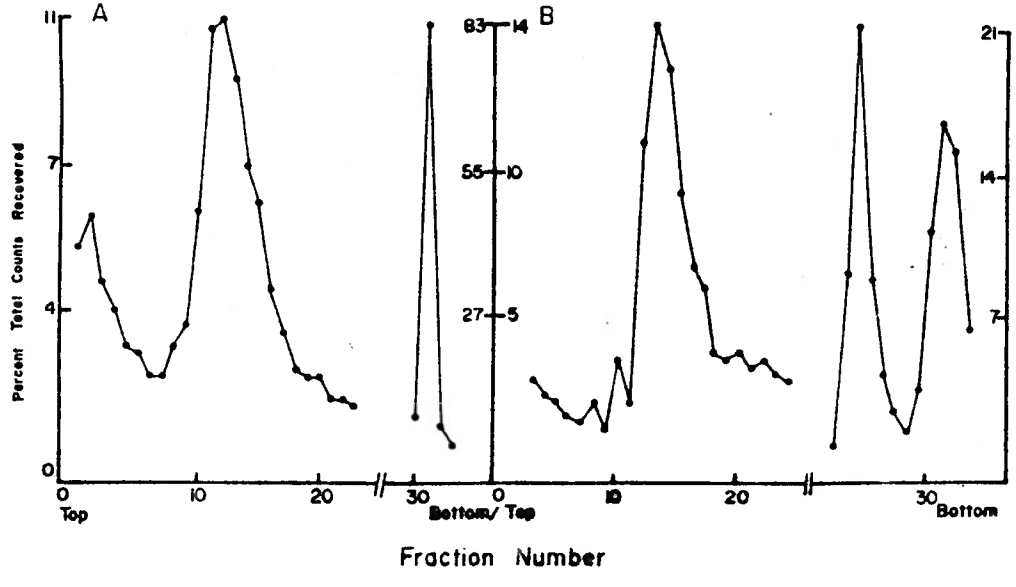
CHAPTER IV

RESULTS

Ninety-percent (90%) of a 32S monomeric species of ColE1-Ap plasmid DNA has been shown to sediment on neutral sucrose gradients (CLOS) free of chromosomal DNA, when the gradients from exponential cultures were selectively centrifuged (45,000 rpm-2 hr-5 C) for the isolation of freely sedimenting plasmid DNA. Treatment of cultures with the antibotic, chloramphenicol (Cm), caused a complete reversal in this distribution and only 10% of the ColE1-Ap plasmid DNA was isolated as freely sedimenting forms (Gosier, 1979) (Figs. 2A and B).

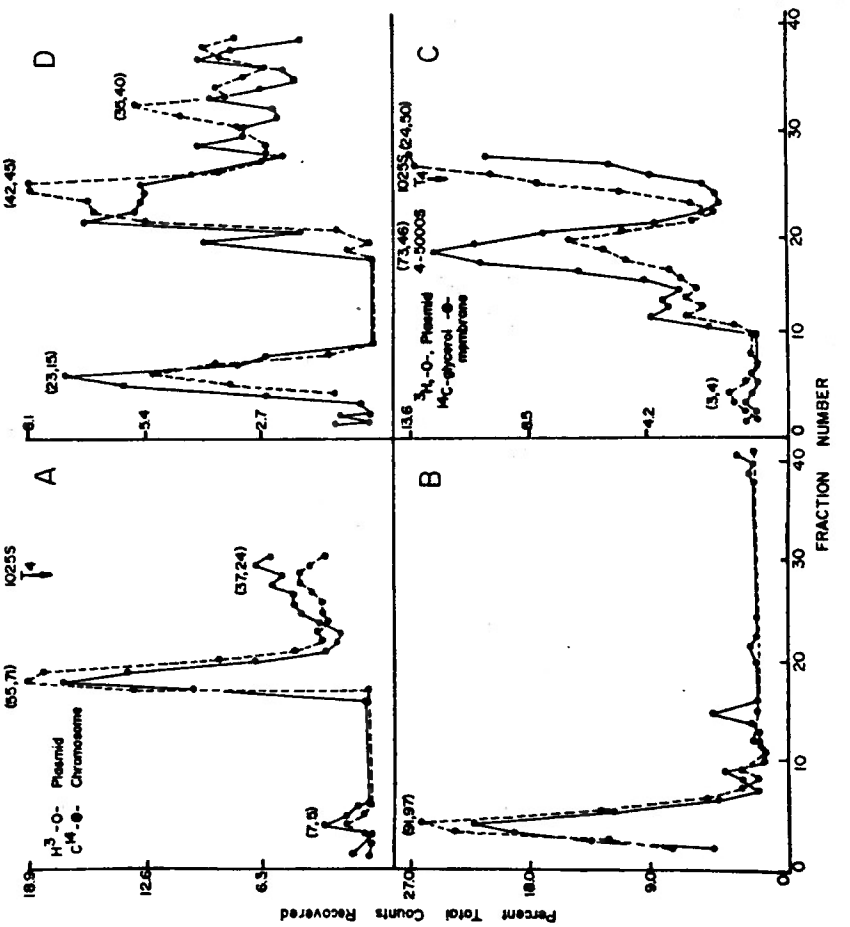
Slow speed centrifugation has been employed to facilitate the isolation of bulk bacterial nucleoids (chromosomal DNA, plasmid DNA and membrane fragments). Nucleoids isolated from exponentially grown cultures sediment in the 3200S region of the gradient (Ryder and Smith, 1974). Cultures treated with Cm revealed a shift in the sedimentation value of the nucleoid to 5000S, which is well in the region of a highly-folded-membrane-associated chromosome (Gosier, 1979). Both exponential and chloramphenicol exposed nucleoids have been analyzed to determine the intracellular localization of ColE1-Ap plasmid DNA.

Fig. 2. Typical profiles of ColE1-Ap plasmid DNA prepared by the CLOS procedure. Exponential and chloramphenicol treated cultures, A and B, respectively, were labeled with [^{3}H]-dThd. Centrifugation was at 45,000 rpm for 2 hr at 5 C. (●——●), [^{3}H]-dThd.


A
B
Percent Total Counts Recovered
11
7
4
0
83
55
27
14
10
5
21
14
7
0
10
20
30
0
10
20
30
Top
Bottom/ Top
Bottom
Fraction Number

The 3200S bacterial nucleoid from exponential cultures, as well as the 5000S species from Cm incubated cultures were subjected to the CsCl-sucrose gradient of Sueoka and Hammers (1974). These gradients were designed to separate free DNA from membrane associated DNA from free membrane on the basis of their respective densities of 1.71 g/cc, 1/35 g/cc, and 1.21 g/cc. Cultures of RS86 were equilibrated 30 min, then prelabeled with 1 μCi/ml [^{14} C]-glycerol to label membrane components and divided into two aliquots. Both cultures were monitored until an optical density of 0.3 was achieved. One exponential culture was then pulse-labeled for 1 min with 30 μCi/ml [^{3} H]-dThd and rapidly poured over crushed KCN to inhibit metabolic activities. Chloramphenicol (200 μg/ml) was added to the second culture portion and incubation continued for 2 hr. Afterwards, the Cm culture was pulse-labeled with 30 μCi/ml of [^{3} H]-dThd for 1 min and harvested over crushed KCN. Figures 3A and C are representative profiles of CLOS gradients where the bulk nucleoids were isolated. Approximately 55% and 73%, respectively, of the radioactive counts were recovered in plasmid molecules co-sedimenting with the membrane-associated chromosome. When the exponential lysate of the nucleoid material was pooled, dialyzed against buffer, vortexed, and centrifuged for CsCl-sucrose analysis, the distribution of pulsed DNA occurred as illustrated in Fig. 3B. Ninety-one percent (91%) of the pulsed DNA counts from exponential cultures band in the free DNA region. However, after Cm incubation, 42% (Fig. 3D), of the pulsed plasmid counts band in the membrane-associated region

Fig. 3. CLOS gradient profiles and profiles of CsCl-sucrose double density gradients. For CLOS profiles A and B, exponential cultures were prelabeled with [^{14}C]-dThd and [^{14}C]-glycerol, respectively. See Methods section for centrifugation conditions for CsCl-sucrose double gradients (B and D). Conditions for pulse-labeling are stated in the text. Sedimentation and density increases from right to left.


A
B
C
D
Plasmid
Chromosome
(65,71)
(37,24)
(7,8)
(91,97)
(23,15)
(42,43)
(35,40)
(73,46)
(24,30)
(3,4)
FRACTION NUMBER
Percent Total Counts Recovered

and only 23% were isolated as free DNA. A small percent (35%) of plasmid counts were unexpectedly isolated in the free membrane region, possibly representing nonspecifically bound DNA released during preparative procedures (Sparks and Helinski, 1979).

The preparative procedures for CsCl-sucrose gradient analysis greatly influenced the stability of membrane-plasmid DNA associations. The stability of amplified ColE1-Ap-membrane associations was investigated over prolonged labeling periods. Culturing techniques and pulse labeling were performed as previously mentioned; however, equal aliquots were removed from the Cm culture after 30 s, 1 min, 10 min, 60 min, 120 min, and 240 min incubation periods. Nucleoids were isolated and each pooled sample was divided in half. One-half was vortexed and the other remained untreated. Data in Table 1 suggest that a very high degree of membrane-plasmid complexing occurred during the short pulse of 30 s (85%) and 1 min (75%) for unvortexed cultures. The plasmid DNA (40%) from unvortexed samples remained membrane bound up to 4 hr after labeling. High degrees of membrane associations were observed in vortexed samples derived from 30 s (46%) and 1 min (50%) aliquots. A dramatic decrease was observed in this membrane-associated DNA (3%) after 4 hr of labeling. Vortexing, as suggested by these data, probably shared plasmid DNA from its membrane maintenance template.

An isogenic F^- strain of E. coli, RS46, was grown and labeled under exponential conditions. When bulk nucleoids from this preparation were analyzed by the CSCL-sucrose gradient technique,

Table 1. Percentage distribution* of [^{3}H] counts in ColE1-Ap plasmid DNA on cesium chloride-sucrose double gradients with (+) and without (-) vortexing of samples.

		% Distribution		
Sample	Vortex	B[1]	M[2]	T[3]
30s	+	35.0	46.0	19.0
	-	6.0	85.0	9.0
1 min	+	26.0	50.0	24.0
	-	10.0	75.0	15.0
10 min	+	68.0	22.0	10.0
	-	46.0	40.0	14.0
60 min	+	87.0	7.0	6.0
	-	38.0	59.0	3.0
120 min	+	91.0	6.0	3.0
	-	47.0	51.0	2.0
240 min	+	95.0	3.0	2.0
	-	58.0	40.0	2.0

[1]Bottom of gradient - free DNA region.

[2]Middle ofgradient - DNA-membrane associated region

[3]Top of gradient - free membrane region

*Described in Methods.

the data indicated that 95% of the chromosomal DNA from the culture band as free DNA (data not shown). The growth curve of the F^- strain (Fig. 4) and the analysis of radioactive uptake (Fig. 5) illustrate the complete cessation of growth and radioactive incorporation approximately 60 min after addition of the antibiotic, Cm.

Experimentation was devised to pinpoint the specific membrane fraction involved in the association of ColE1-Ap DNA. The 30-50% step neutral sucrose equilibrium gradient, developed by Osborn et al. (1974), selectively separated outer membrane fractions from cytoplasmic membrane fractions on the basis of their density, 1.22 g/cc and 1/16 g/cc, respectively. Nucleoids prepared from exponential and Cm cultures were pooled, dialyzed, and centrifuged on discontinuous sucrose gradients. The results are illustrated in Fig. 6. Figure 6 A depicts the distribution of pulsed counts from exponential cultures. Eighty-seven percent (87%) of the DNA counts band with 77% of the labeled membrane in the outer membrane (OM) region of the gradient designated as H_1 and H_2. Thirteen percent (13%) of the pulsed counts band in the inner or cytoplasmic membrane (IM) region, represented as L_1 and L_2. Figure 6 B represents the results obtained after Cm amplification of plasmid DNA. The majority (94%) of the [^{3}H]-dThd plasmid DNA counts repeatedly band in the IM region, along with 94% of prelabeled membrane. To substantiate that the plasmid molecules in the IM region were indeed membrane-associated and not freely sedimenting DNA forms, a second discontinuous gradient was employed. The body of this gradient was 20-40% sucrose

Fig. 4. A growth curve for strain RS46 in the presence (●———●) and absence (O———O) of Cm. Growth conditions are listed in the text.

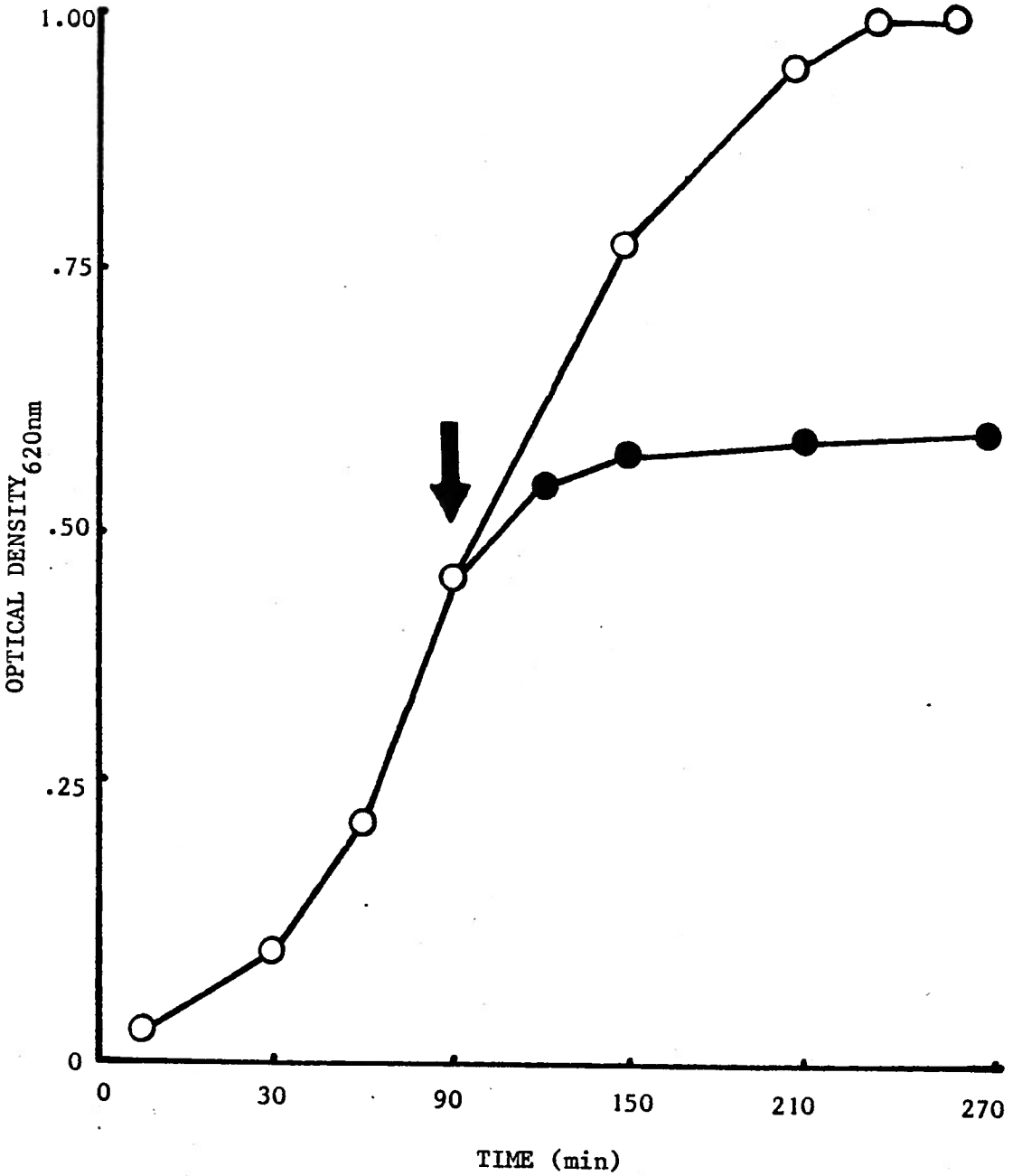

1.00
.75
.50
.25
0
OPTICAL DENSITY 620nm
0
30
90
150
210
270
TIME (min)

Fig. 5. A graph of the uptake of [^{3}H]-dThd in the absence (O——O) and presence (●——●) of Cm. Labeling conditions are listed in the text.

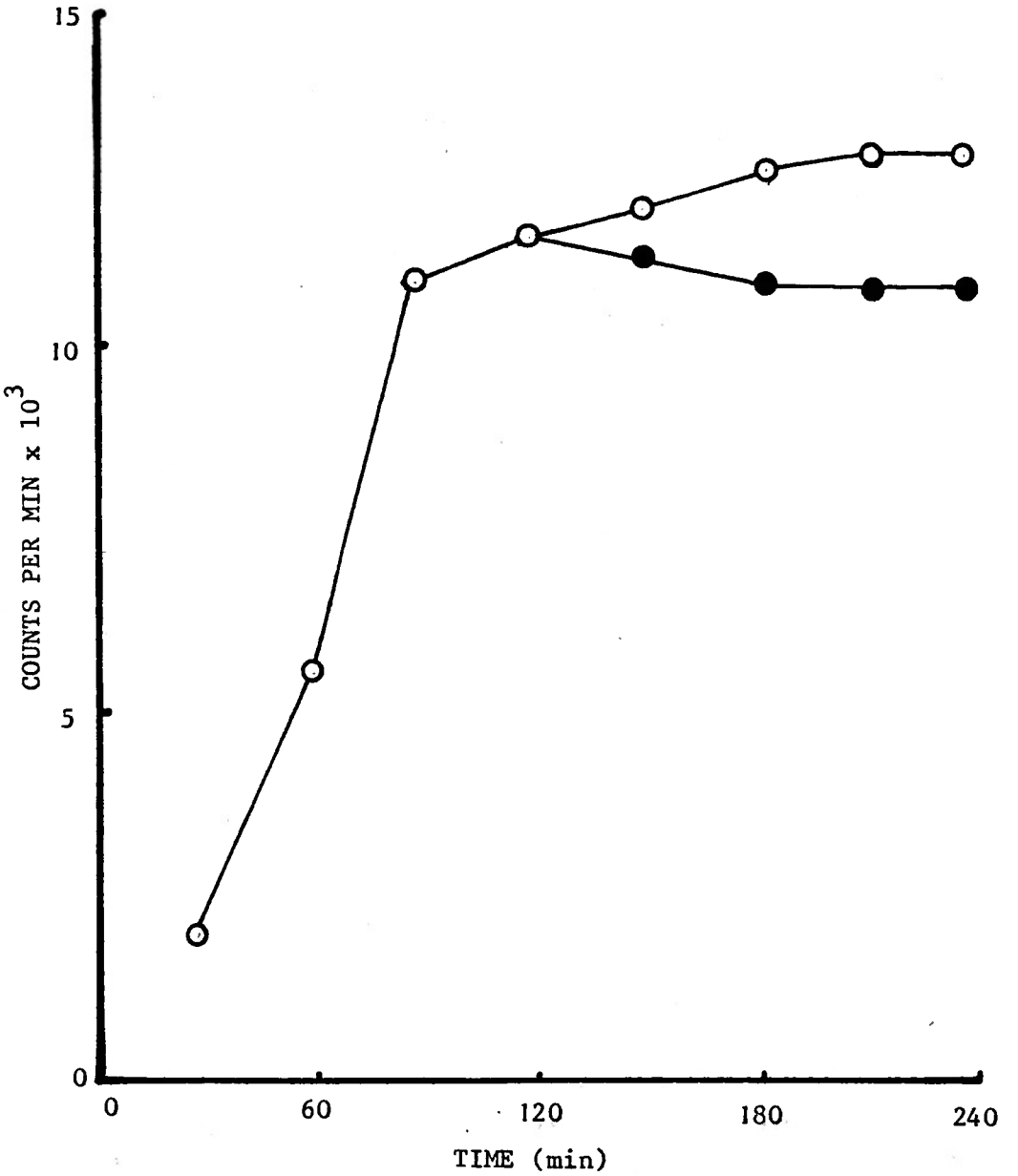
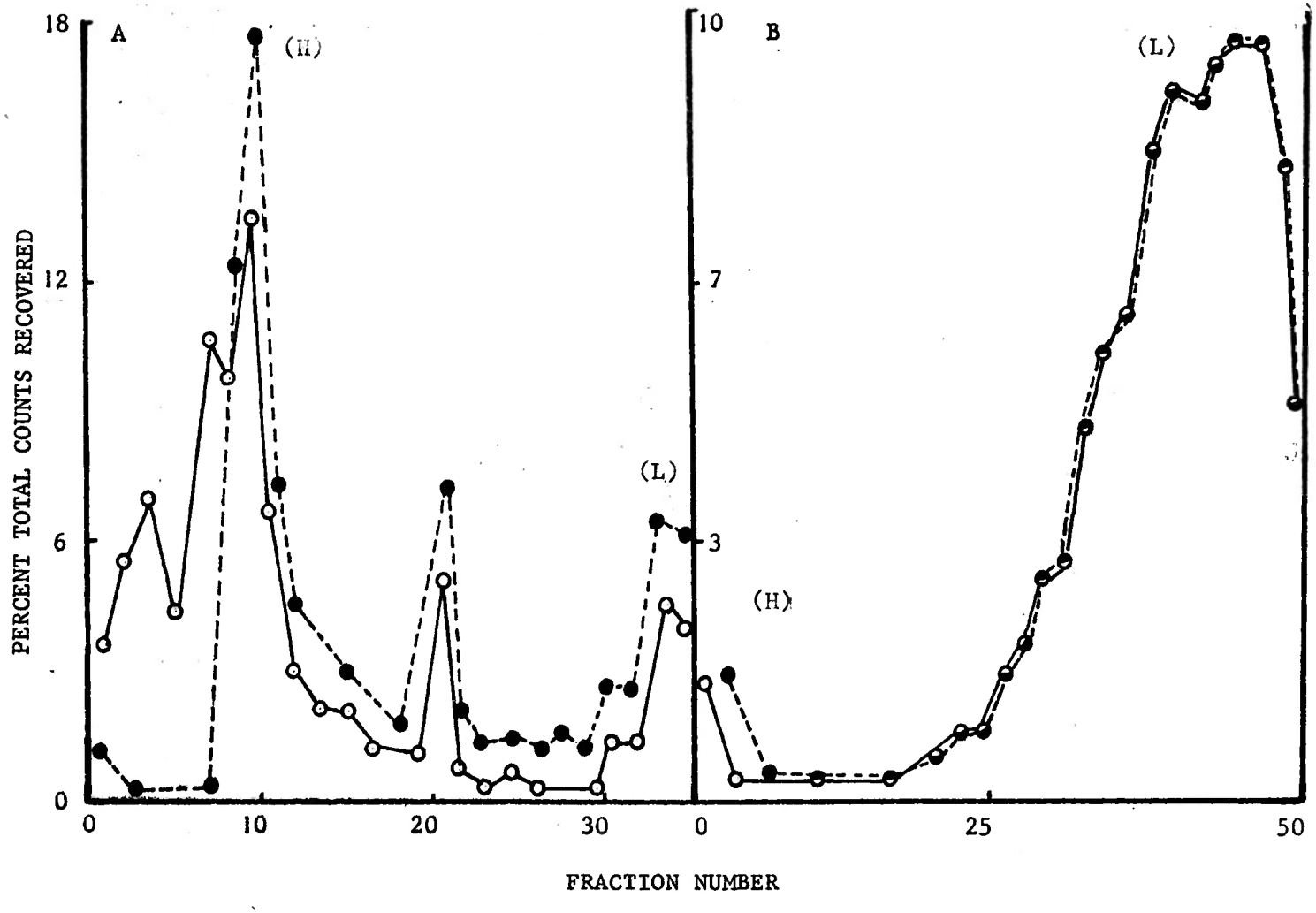

15
10
5
0
COUNTS PER MIN x 10^3
0
60
120
180
240
TIME (min)

Fig. 6. Profiles of 30-50% isopycnic step neutral sucrose gradients. Nucleoid samples isolated from exponential (A) and Cm treated cultures (B) prelabeled with [^{14} C]-glycerol (●——●) were prepared for centrifugation as described in the Methods section. Centrifugation was at 36,000 rpm for 18 hr at 5 C. Density and sedimentation increase from right to left.


A
B
(H)
(L)
(L)
(H)
18
12
6
0
10
7
3
0
10
20
30
0
25
50
PERCENT TOTAL COUNTS RECOVERED
FRACTION NUMBER

and therefore, predominantly of IM density. Data in Fig. 7 indicate that 99% of the plasmid counts isolated from IM regions of the 30-50% gradient band toward the center of the 20-40% gradient with 99% of the membrane glycerol counts at a density of 1.16 g/cc. These results further illustrated a specificity in the association of ColE1-Ap plasmid DNA and inner membrane fragments.

Correlative studies were performed on an isogenic F^- strain of E. coli (data not shown). Studies revealed that 89% of the [^{3} H]-dThd labeled chromosomal DNA counts from exponential cultures band in the CM region, similar to reports by Wolf-Watz et al. (1979), while 11% of the pulsed counts were found in the IM region. After Cm incubation the majority of [^{3} H]-dThd counts remained in the Cm region (72%). The 27% found associated in the IM region possibly represented small amounts of replication fork DNA, as discussed by Nicolaidis and Holland (1978). Their data specifically illustrated that the predominant bacterial chromosomal DNA binding site is Cm. Hence, the high percentage of IM associated tridium counts isolated upon analysis of Cm treated RS86 in the present study is a direct result of ColE1-Ap associations.

Table 2 summarizes the results obtained when bacterial nucleoids were centrifuged on the step neutral gradient. Distributions of radioactive counts from H_1 plus H_2 (heavy outer membrane) and L_1 plus L_2 (light inner membrane) were combined. Nucleoids isolated from exponential lysates of RS86 and the isogenic F^- strain, RS46, revealed high percentages of association in the outer membrane (CM) region of the gradient, approximately 87% and 89%, respectively.

Fig. 7. Profile of 20-40% isopycnic step neutral sucrose gradient. Chloramphenicol treated ColE1-Ap associated inner membrane fragment complexes were isolated as described in Methods and recentrifuged on the gradient. Inner membrane fragments were labeled with [^{14}C]-glycerol (●——●) and ColE1-Ap DNA with [^{3}H]-dThd (O——O).

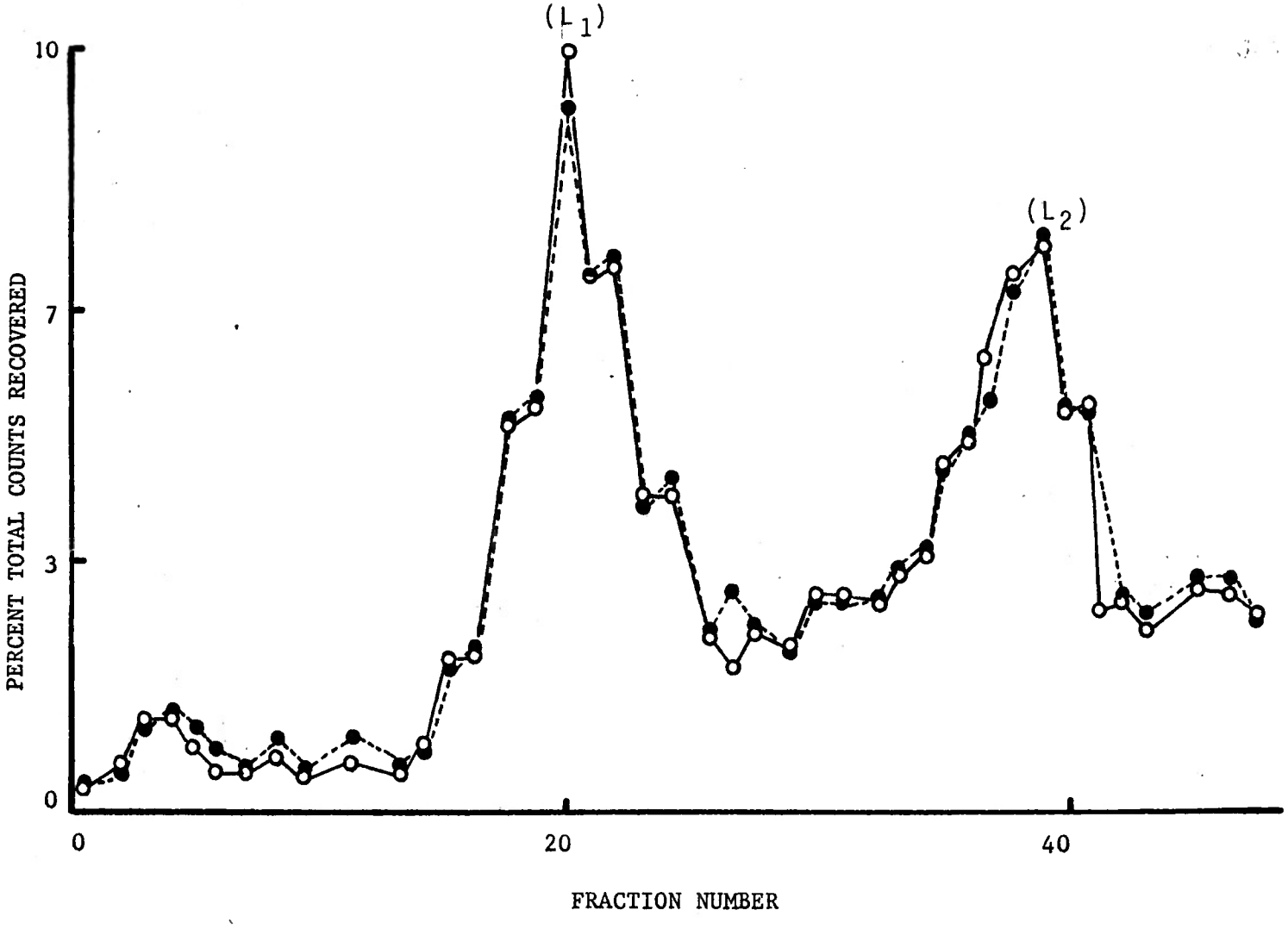

(L1)
(L2)
10
7
3
0
PERCENT TOTAL COUNTS RECOVERED
0
20
40
FRACTION NUMBER

Table 2. Percentage distribution of CLOS nucleoid counts isolated from 50-30% step neutral gradients.

Experiment	Percent Distribution - ^{3}H, ^{14}C-glycerol	
	$H_1 + H_2$ [1]	$L_1 + L_2$ [2]
Exponential		
A. Nucleoid (RS86)	87.31, 77.68	12.68, 22.31
B. Nucleoid (RS46)	89.03, 95.06	10.96, 4.13
Chloramphenicol		
C. Nucleoid (RS86)	4.61, 5.12	95.30, 94.07
D. Nucleoid (RS46)	72.07, 70.13	27.12, 21.06
E. Nucleoid (RS86)[3]	- -	99.19, 99.99

[1] $H_1 + H_2$ = Outer membrane

[2] $L_1 + L_2$ = Inner membrane

[3] $L_1 + L_2$ isolated from 40-20% gradients

After incubation in chloramphenicol, approximately 95% of the tridiated molecules (ColE1-Ap) band in the inner membrane region of the gradient while the majority (72%) of the chromosomal DNA from the F^- strain remained in the outer membrane region. The non-labile association of the Cm incubated plasmid DNA was substantiated by a second (20-40%) step neutral sucrose gradient. More than 90% of the ColE1-Ap plasmid DNA molecules remained in strict association with more than 90% of the inner membrane fragments when subjected to the second centrifugation.

Analysis of the distribution of freely sedimenting forms of ColE1-Ap plasmid DNA on the 30-50% step neutral sucrose gradient is depicted in Table 3. The plasmid DNA region, as well as the chromosomal DNA region were separately pooled and dialyzed against buffer. Centrifugation of the exponential plasmid DNA resulted in a large majority of inner membrane association, represented as 53.48% in the L_1 region of the gradient and 22.49% in the L_2 region. Similarly, centrifugation of Cm incubated plasmid DNA on a similar gradient revealed high coincidence of association between ColE1-Ap plasmid DNA and inner membrane fragments. Plasmid DNA pulsed for 1 min with [^{3}H]-dThd revealed association distributions of 18.32% and 49.03% in the L_1 and L_2 regions of the gradient, respectively. After a 30 min exposure to label, the majority of the ColE1-Ap plasmid DNA molecules remained associated with inner membrane fragments, represented as 19.44% in the L_1 region and 61.77% in the L_2 region of the gradient. Data in Table 3 also suggest that the majority of tridiated counts (67.17%) from the chromosomal region of exponential

Table 3. Percentage distribution of freely sedimenting plasmid DNA and chromosomal DNA counts from CLOS gradients isolated on 50-30% step neutral gradients.

Experiment		Percent Distribution - ^{3}H, ^{14}C-glycerol			
		H_1	H_2	L_1	L_2
Exp					
1	plasmid[1]	11.57, 19.68	12.45, 13.47	53.48, 35.92	22.49, 3091
2	chromosome[2]	67.17, 55.22	- -	19.49, 30.41	18.33, 14.36
Cm					
3	plasmid (1' pulse)	19.23, 22.24	13.23, 20.28	18.32, 17.37	49.03, 40.09
4	plasmid (30' pulse)	10.07, 15.85	8.70, 22.18	19.44, 20.43	61.77, 41.52
5	chromosome (1' pulse)	11.18, 18.30	11.48, 15.73	52.52, 33.99	2.41, 31.97
6	chromosome (30' pulse)	0.78, 6.19	12.63, 21.91	6.79, 21.41	79.79, 50.47

[1]Plasmid DNA isolated from plasmid peak of long term CLOS gradient.

[2]Chromosomal DNA isolated from bottom of long term CLOS gradient.

gradients band in the outer membrane (H_1) region of the step neutral sucrose gradient. On the contrary, after amplification with the antibiotic chloramphenicol, only 11.18% of the pulsed (1 min) counts from the chromosomal DNA region of the CLOS gradient band with outer membrane fragments on the step gradient. Fifty-four percent (54%) of these pulsed counts were isolated in the inner membrane region; 52.52% in the L_1 region and 2.41% in the L_2 region of the gradient. Thirty min after the pulse label, only 6.79% of these counts remained associated with L_1 membrane fragments, however, 79.79% of the pulsed counts were isolated in the L_2 membrane region of the gradient. All cultures labeled after Cm incubation reveal distributions of [^{3} H]-dThd ColE1-Ap DNA, predominantly. These types of comparative studies suggest that there is a prominent association between Cm-treated ColE1-Ap plasmid DNA and inner membrane fragments on step neutral sucrose gradients.

Experimentation was designed to illustrate the involvement of specific membrane-associated proteins in the complexing of ColE1-Ap plasmid DNA molecules to the bacterial cell membrane fragments. The sodium dodecyl sulfate polyacrylamide gel electrophoretic system (SDS-PAGE) of Lammeli et at. (1976) was employed toward this end. The outer membrane regions and cytoplasmic membrane regions were separately pooled from step neutral sucrose gradients. These samples were mixed and solubilized for 1.5 min at 60 C in buffer containing 0.0625 M Tris-HCL, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue (as the tracking dye). Approximately 50-100

µl of sample was layered onto the stacking gel (3%) of a 10% SDS polyacrylamide gel. Electrophoresis was performed until the tracking dye was approximately 1 cm from the bottom of the gel. Figure 8 represents electrophoretic results obtained from samples of exponential and Cm incubated nucleoids of RS86. An array of polypeptide bands, ranging from MW 14,000-48,000 was discernable in each gel track. No apparent difference was illustrated in recoverable bands from either culture type. The inner and outer exponential and Cm-treated membrane proteins of the F^- strain (RS46) were surveyed to provide a comparative study with the membrane proteins of the ColE1-Ap containing strain. Separated membrane isolates of RS46 were prepared and electrophoresed as previously indicated. The protein banding patterns are indicated in Fig. 9. Fifteen (15) bands were discernable in tracks 1 and 2, containing inner membrane polypeptides from exponential and Cm-treated inner membranes, respectively. These proteins ranged in molecular weights from 14,000 to 60,000. Gel tracks 3 and 4 contained membrane proteins obtained from exponential and Cm-incubated outer cell membranes, respectively. Twelve (12) protein bands were visible in each track. A very prominent band was recovered, with an apparent MW between 20-24,000, from both culture types.

The isolated cytoplasmic and outer membrane proteins of exponential and amplified cultures of RS86 are depicted in Fig. 10. Several membrane proteins were isolated from the two culture types of inner membranes (gel tracks 1 and 2). The tracks containing

Fig. 8. Profile of a 10% SDS polyacrylamide gel (SDS-PAGE). Exponential and Cm-treated nucleoids (tracks 1 and 2, respectively) were electrophoresed at 10 mA for 20 hr. Track 3 and the last traci (from left to right) of all subsequent gels contained standard protein markers of MW: 66,000, 45,000, 36,000, 34,000, 24,000, and 14,300. Molecular weights decrease from the top to the bottom of the gel. See the Methods section for preparation of gels and samples.

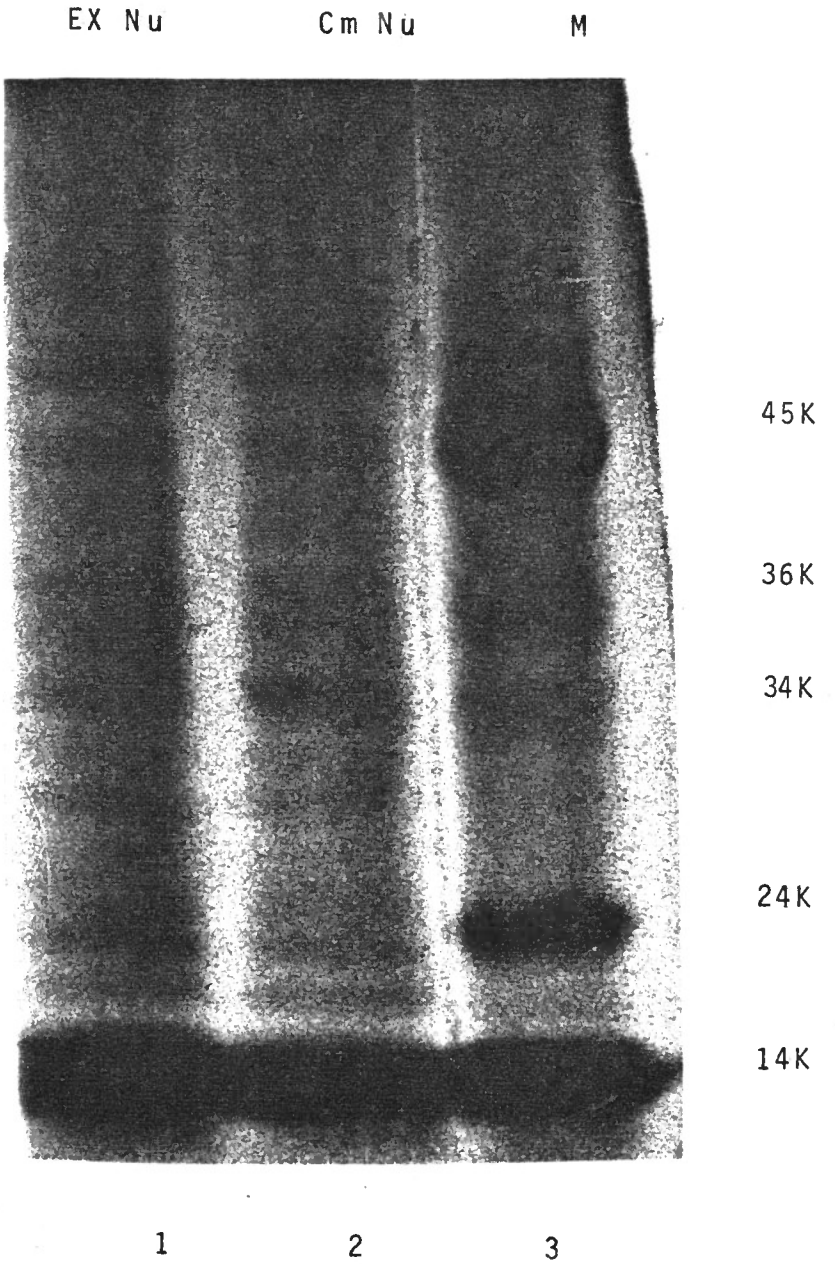

EX Nu
Cm Nu
M
45K
36K
34K
24K
14K
1
2
3

Fig. 9. A 10% SDS-PAGE of proteins isolated from the membranes of the RS46 F^- strain of *E. coli*. Gel tracks 1 and 2 contained inner membrane proteins of exponential and Cm-treated samples. Tracks 3 and 4 reveal the banding patterns of proteins isolated from exponential and Cm treated outer membrane fragments. Track 5 contained the MW markers.

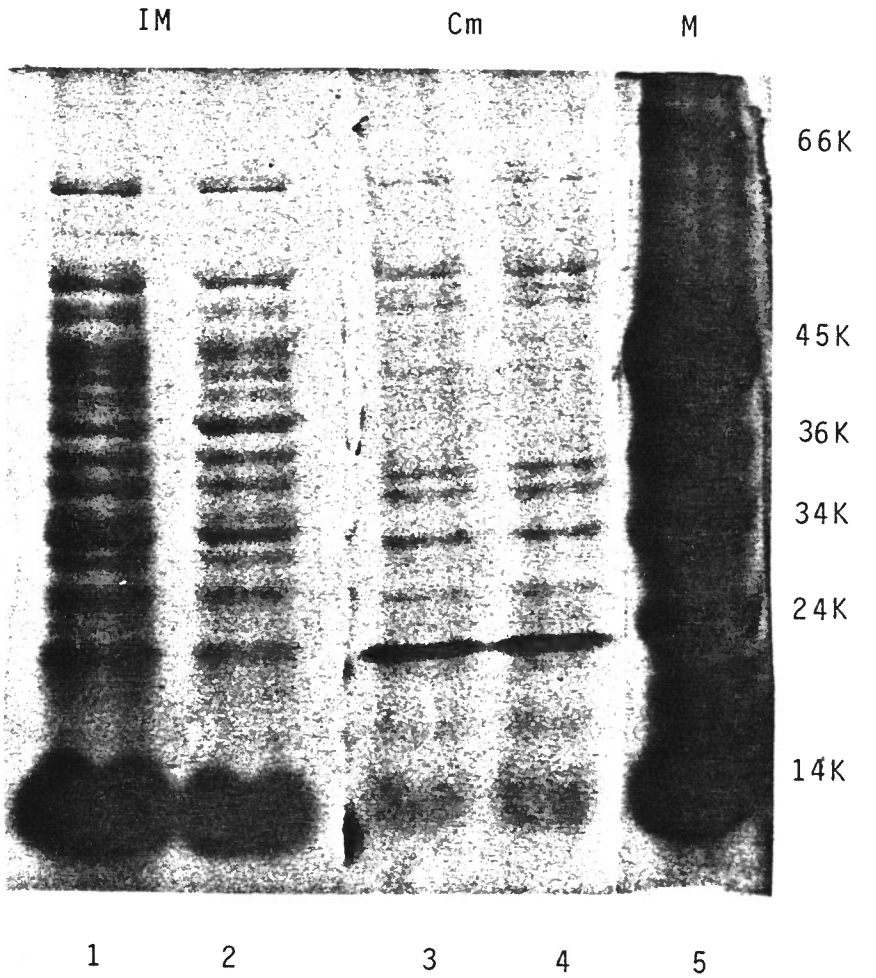
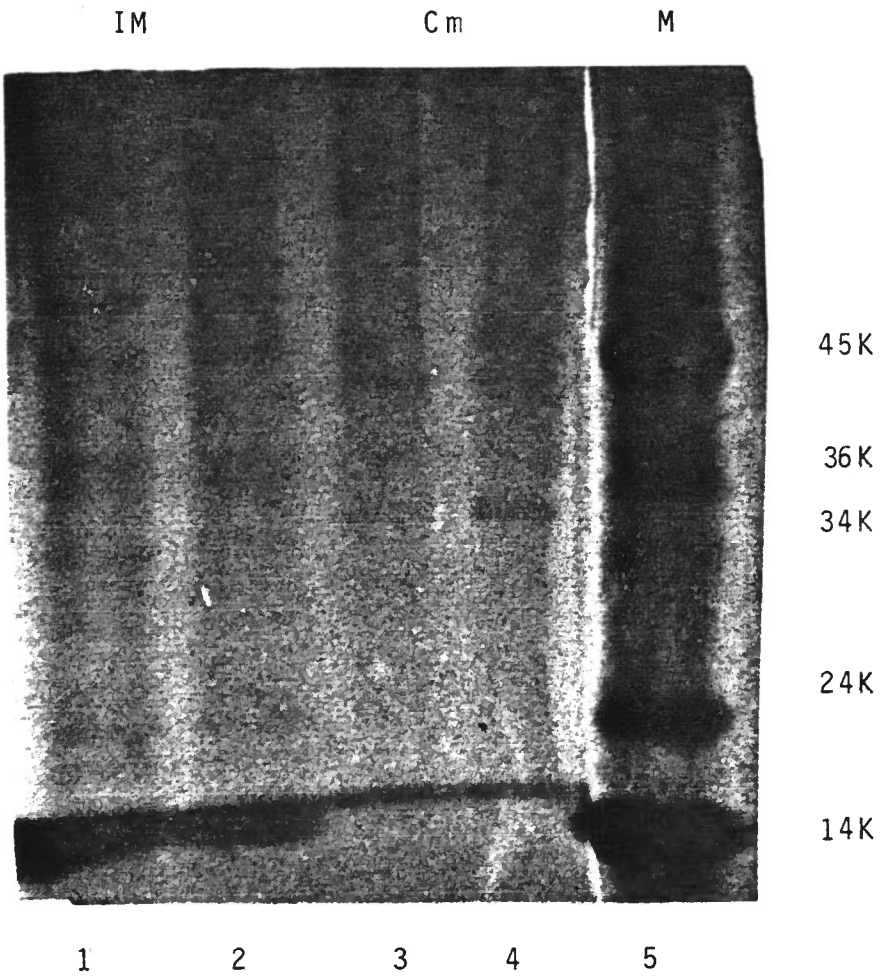

IM
Cm
M
66K
45K
36K
34K
24K
14K
1
2
3
4
5

Fig. 10. A profile illustrating a 10% SDS polyacrylamide gel. The gel tracks contained inner membrane proteins from exponential (1) and Cm-treated (2) samples of strain RS86. Tracks 3 and 4 contained outer membrane proteins from exponential and Cm-treated samples, respectively. Track 5 contained the MW protein markers.


IM
Cm
M
45K
36K
34K
24K
14K
1
2
3
4
5

exponential and Cm-treated outer membrane proteins (3 and 4) revealed only 3 resolvable polypeptides. The approximate molecular weights were 35,000, 44,000, and 46,000, respectively.

Freely sedimenting forms of ColE1-Ap plasmid DNA derived from exponential and Cm treated cultures were subjected to SDS polyacrylamide gel electrophoresis. Figure 11 illustrates the proteins which associated with freely-sedimenting ColE1-Ap molecules. Four (4) proteins were isolated from the 2 culture types, each migrating to a region of molecular weight 36,000, 44,000, and 48,000 in increasing fashion.

Electrophoresis of the proteins isolated from freely-sedimenting plasmid regions along with inner and outer membrane proteins is presented in Figure 12. These profiles suggest that the proteins isolated from the plasmid DNA of CLOS gradients migrated to MW regions which corresponded with similar MW regions (48,000 and 36,000 of inner; 46,000 and 44,000 of outer) of membrane proteins isolated from cultures grown in the presence and absence of antibiotic.

Fig. 11. A 10% gel profile illustrating proteins which co-sedimented with freely-sedimenting forms of ColE1-Ap plasmid DNA. Track 1 depicts proteins associated with exponential isolates of the plasmid. Track 2 represents the proteins that co-sedimented with Cm-treated plasmid DNA. Track 3 contained the MW markers.

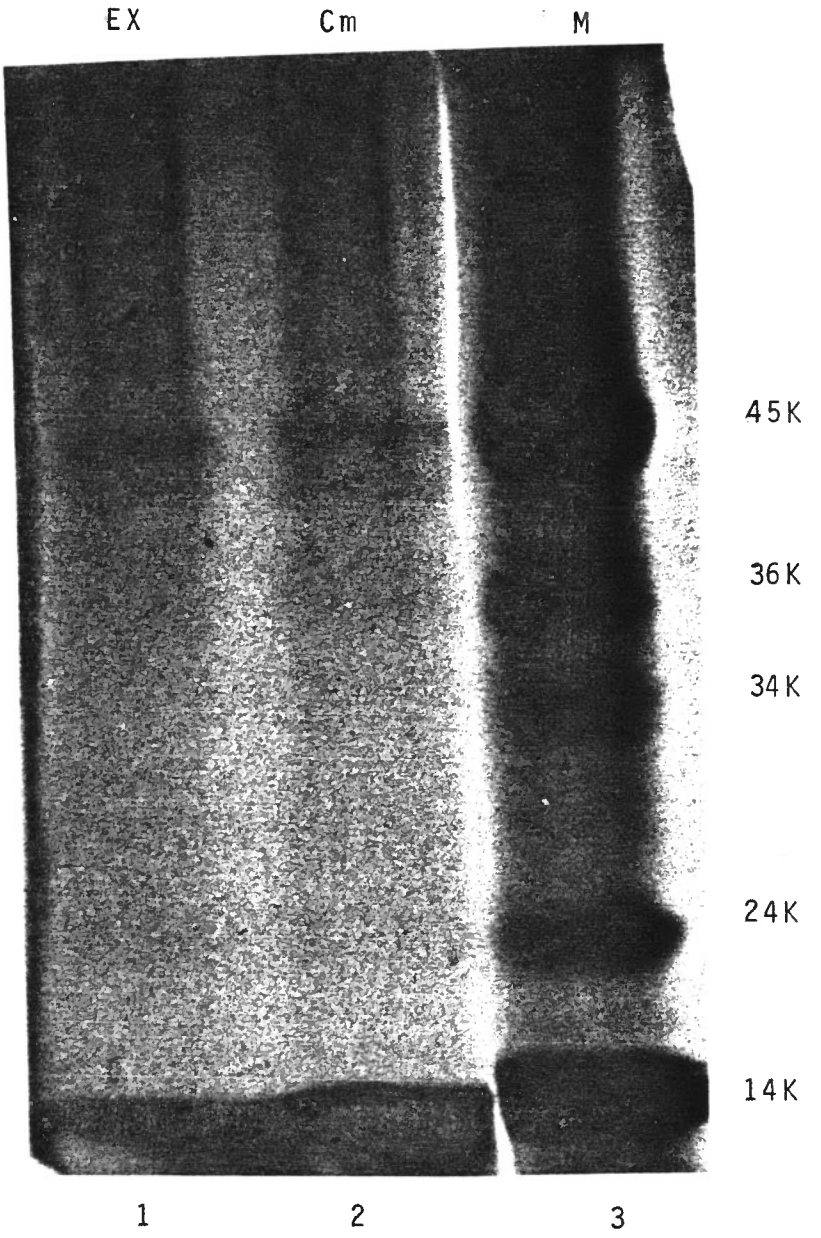
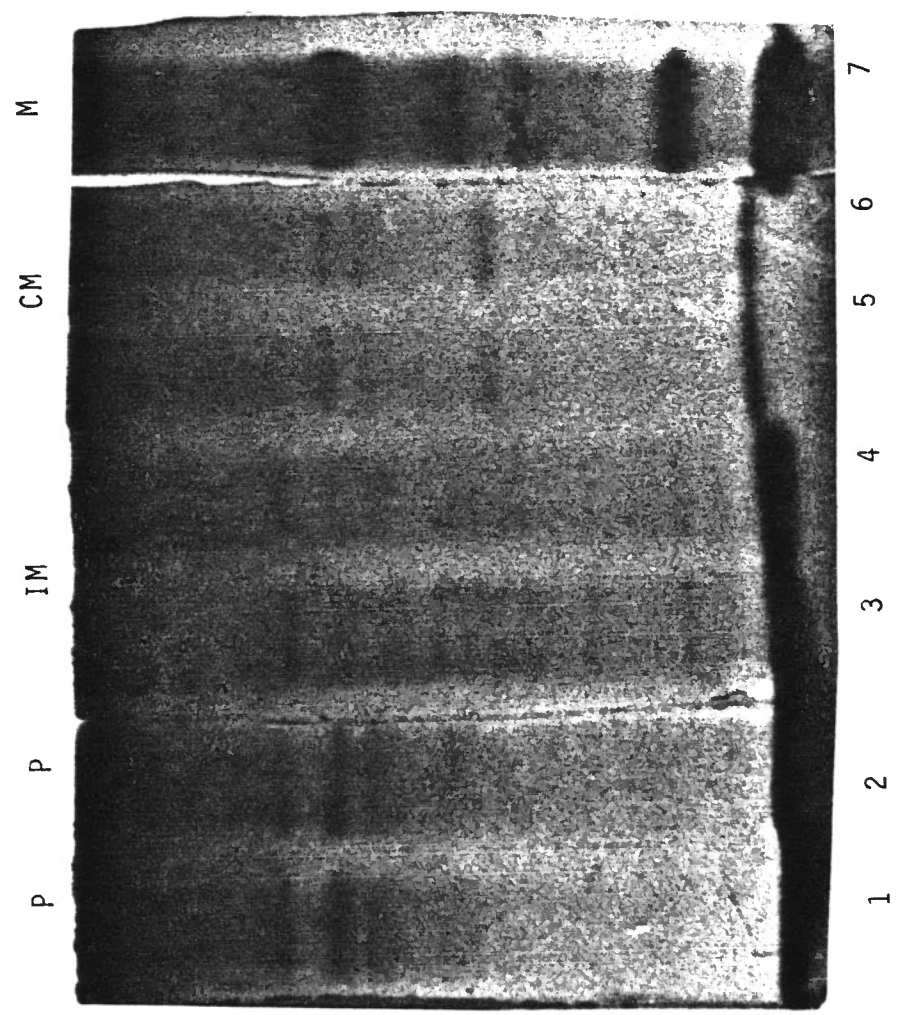

EX
Cm
M
45K
36K
34K
24K
14K
1
2
3

Fig. 12. A gel profile showing cumulative results of protein banding patterns. This illustraion specifically depicts similarities in the migrational patterns of free ColE1-Ap associated proteins and inner and outer membrane associated proteins. Gel tracks 1, 3 and 5 represent ColE1-Ap associated proteins, inner membrane proteins and outer membrane proteins isolated from exponential samples, respectively. Gel tracks 2, 4, and 6 represent ColE1-Ap associated proteins, inner membrane proteins and outer membrane proteins isolated from Cm-treated samples. Track 7 contained the MW protein markers.


45K
36K
34K
24K
14K
P
P
IM
CM
M
1
2
3
4
5
6
7

CHAPTER V

DISCUSSION

Previous data indicated that a singular monomeric species of ColE1-Ap (32S) plasmid DNA was isolated as freely-sedimenting forms on CLOS gradients, selectively centrifuged for such isolations. Ninety percent (90%) of these plasmid molecules isolated under exponential conditions sedimented free of chromosomal DNA-membrane complexes. However, after incubation in chloramphenicol, only 10% of the 32S ColE1-Ap DNA molecules were isolated as freely sedimenting forms, while 90% of the molecules sedimented near the bottom of the gradient in the chromosomal DNA-membrane region (Gosier, 1979) (Fig. 2). This data suggested that exposure to the antibiotic induced increased associations of the plasmid DNA to chromosomal DNA and/or the bacterial cell membrane. These association frequencies are in accord with reports of Kline et al. (1976). Further, it has been postulated that Cm may selectively produce molecules different in molecular configuration than those generated during exponential growth (Sparks and Helinski. 1979). However, our results indicated that the monomeric nature of ColE1-Ap molecules associated with the membrane-folded chromosomal complex during exponential growth was maintained after exposure to chloramphenicol.

Evidence presented by Sheehy et al. (1977) indicated that the amount of monomeric species of R6K plasmid DNA which co-sedimented with the folded chromosomal complex on selective CLOS gradients depended on the conditions of lysis, i.e., low salt, the presence of ribonuclease and protease. Stonington and Pettijohn (1971) have shown that the folded chromosomal complex involved ribonucleic acid (RNA) and protein. These observations suggest that the only restraining factor in the release of monomeric species of plasmid DNA from chromosomal DNA-membrane complexes (nucleoids) was the maintenance of the folded configuration. We have employed a procedure, initially devised by Sheehy and Novick (1975) for the isolation of replicative intermediates in Staphylococcus aureus to isolate bacterial nucleoids form RS86 harboring ColE1-Ap plasmid DNA. Figure 3A illustrates the tight association of [^{3} H]-dThd pulsed ColE1-Ap molecules and [^{14} C]-dThd labeled chromosomal DNA on CLOS gradients. Fifty-five percent (55%) of the pulse-labeled plasmid isolated from exponential cultures sedimented in the nucleoid region along with 71% of the labeled chromosomal DNA. Plasmid DNA (73%) pulsed after Cm incubation also co-sedimented with the bacterial nucleoid, illustrated in Fig. 3C along with 46% of pre-labeled [^{14} C]-glycerol membrane fragments.

The replication of DNA in bacteria, as proposed by Jacob et al. (1963) required attachment to the bacterial cell membrane. Evidence from minicell preparations of E. coli has indicated membrane ColE1 associations from a region including the origin/terminus

of DNA replication (Sparks and Helinski, 1979). Data presented in Fig. 3 indicated a preferential association of amplified ColE1-Ap with membrane component on CsCl-sucrose gradients, rather than with the bulk of host folded chromosomal DNA (Miller et al., 1978). Investigation of the intracellular localization of pulse-labeled ColE1-Ap molecules after amplification in Cm.

The replication of DNA in bacteria, as proposed by Jacob et al. (1963) required attachment to the bacterial cell membrane. Evidence from minicell preparation of E. coli has indicated membrane-C.1E1 associations from a region including the origin/terminus of DNA replication (Sparks and Helinski, 1979). Data presented in Fig. 3 indicated a preferential association of amplified ColE1-Ap with membrane component on CsCl-sucrose gradients, rather than with the bulk of host folded chromosomal DNA (Miller et al., 1978). Investigation of the intracellular localization of pulse-labeled ColE1-Ap molecules after amplification in Cm, yielded results depicted in Fig. 3D. The data revealed a high level (42%) of membrane-bound pulse-labeled plasmid DNA from cultures prelabeled with [^{14}C]-glycerol. Cultures grown in the presence of glycerol increase rapidly in turbidity and have been proposed to contain increased numbers of membrane templates (membrane sites) which may account for the high level of membrane-associated pulsed molecules. Additional evidence to support this postulate was provided via cesium chloride-ethidium bromide (CsCl-EtBr) equilibrium density gradient centrifugation. An increase, from 4 to 22% of covalently

closed (CCC) ColEl-Ap molecules generated from deoxythymidine and glycerol grown cultures, respectively, was observed (Gosier, 1979).

The banding pattern of the exponential nucleoid from the isogenic F^- strain (RS46) of E. coli was also surveyed on the CsCl-sucrose gradient. Ninety-five percent (95%) of the chromosomal DNA from this strain band in the free DNA region of these gradients. These data further illustrate that the majority of exponential chromosomal DNA molecules are unassociated. The small percent of DNA molecules isolated with membrane fragments probably represents the origin and replication fork DNA (Nicolaidis and Holland, 1978). Verification of the normal logarithmic growth pattern of RS46 is illustrated in Fig. 4. Cessation of growth in the culture occurred approximately 60 min after exposure to Cm. Further, complete inhibition in the uptake of [^{3} H]-dThd by the chromosomal DNA was apparent 60 min after treatment with the antibiotic (Fig. 5). These observations were similar to those obtained from the RS86 ColEl-Ap containing strain where growth was inhibited approximately 95 min after Cm exposure, with a concomitant inhibition of radioactive incorporation. The minimum radioactive incorporation observed resulted from the replication of the plasmid DNA (Gosier, 1979).

Variation in the preparative procedures for CsCl-sucrose gradient analysis revealed intriguing distributions relative to the stability of amplified ColEl-Ap-membrane associations. Data in Table 1 indicate that 40% of the recoverable ColEl-Ap molecules

remained membrane-bound after 4 hr of labeling. However, these molecules were sheared from their membrane templates by vortexing (3% remained complexed after 4 hr). A priori, we suggest that ColE1-Ap DNA initiates, elongates, and completes replication on membrane templates in the presence of Cm. A protein releasing factor was probably responsible for releasing completed molecules from their replication site/s, which apparently occurs during vegetable growth. The de novo synthesis of the releasing factor/s was probably inhibited in the presence of Cm. We suggest that during Cm exposure, ColE1-Ap DNA molecules accumulate on membrane templates as "a bunch of grapes on a vine." This "grapeing effect" may explain the sensitivity of the plasmid DNA to the shear forces of vortexing.

Three (3) morphologically distinct layers exist in the cell membrane of gram-negative bacterial cells (Shands, 1965). These layers include the cytoplasmic membrane, a second membranous structure, the outer membrane at the outer surface of the cell and a peptidoglycan layer between the two. Osborn et al. (1972) developed the step neutral sucrose equilibrium gradient, which they utilize to separate the outer cell membrane fragments from cytoplasmic membrane fragments of Salmonella typhimurium based on density differences. This gradient has been utilized by several investigators as an index to determine the specific membrane fraction involved in the association of the bacterial chromosomal DNA during replication and/or segregation. Evidence has been presented which

indicate that both the chromosomal origin and the chromosomal replication point are associated with a membrane-like structure (Lundqvist-Parker and Glaser, 1974; Nicolaidis and Holland, 1978). Various techniques have been used to isolate the membrane complex of E. coli which contains both cytoplasmic and outer membrane components. Results presented in Fig. 6 reveal that labeled DNA associated with both outer and cytoplasmic membranes after separation of membranes by isopycnic sucrose density gradient centrifugation. The percent distribution of tridiated counts from exponential gradients was prominent in the outer membrane region of the gradient (87%). However, the majority (95%) of pulsed molecules from amplified cultures aggregated on the cytoplasmic membrane fractions of the gradient. Experimentation devised to negate non-specific associations of amplified plasmid DNA with the cytoplasmic membrane required the use of a 20-40% isopycnic gradient. The recentrifugation of the inner membrane material not only illustrated the specific association of amplified ColEl-Ap molecules, but selectively separated the fractions into specific inner membrane components (L_1 and L_2). It is, therefore, likely that the observed interaction between inner membrane and amplified plasmid DNA is not artifactual in nature. Treatment of E. coli cells with antibacterial agents that interfere with protein synthesis (i.e., chloramphenicol or rifampin) supposedly results in a condensation of the nucleoplasm and a concomitant release of DNA from its membrane sites (Dworsky and Schaeter, 1973; Wolf-Watz and Norqvist, 1979). It is recognized

that inhibition of protein synthesis by Cm may have altered the maintenance template/s of polymerizing ColE1-Ap DNA. Studies performed by Norvick et al. (1980) and Nordstrom et al. (1980) may have some relevance in this regard. They suggested that distortions of the division-partitioning process may have led to a high degree of DNA-membrane complexing. Although our data do not refute this contention, we do not believe collapsing of membrane vesicles has led to entrapment of plasmid DNA. However, amplification in Cm may have created "trapped replicative intermediates" containing anomalies preventing their release from membrane templates (Backman et al., 1978).

The observation that amplified plasmid DNA band in the inner membrane region of the isopycnic gradient aroused questions about the behavior of purified plasmid DNA on those gradients. Table 2 illustrates results which substantiate that the majority of freely sedimenting plasmid DNA, isolated from amplified as well as exponential cultures, is mostly distributed among inner membrane fragments. This data elevated the hypothesis that plasmid DNA associates with the bacterial cell membrane possibly via inner membrane fragments during segregation, as suggested by Sparks and Helinski (1979), and during replication since Cm treated cells cease to divide after extensive segregation exposure. High percentages of tridiated DNA counts were isolated with outer membrane fragments from exponential gradients. However, the majority of plasmid DNA molecules that co-sedimented with the chromosomal DNA

on CLOS gradients, after amplification, band with cytoplasmic membrane on the isopycnic gradient. These observations lend credence to one postulate that ColE1-Ap plasmid molecules may attach to specific sites on the bacterial cell membrane during exponential growth. It appears that amplified molecules aggregate on the membrane via a template that is probably maintained by forces other than non-ionic ones, which would be disrupted by detergents used during lysis. The role for the outer membrane in DNA replication has been proposed on the basis of one study which indicated that the origin of chromosomal replication is associated with outer membrane (Nicolaidis and Holland, 1978). The role of cytoplasmic membrane and/or outer membrane involvement in plasmid DNA replication may be postulated, but evidence toward this end has not been presented.

Wolf-Watz et al. (1978) have found that labeled outer membrane DNA comigrated with one protein having a molecular weight of 31,000 (31K protein) after separation of the outer membrane proteins by SDS-polyacrylamide gel electrophoresis. This protein was covalently linked, since heating to 100 C in SDS-containing buffer was not observed to disassociate the 31K protein from DNA (origin DNA). Experimentation was devised to survey the protein banding patterns of separated and amplified inner and outer membrane fragments, using SDS-polyacrylamide gel electrophoresis (Lammeli and Favre, 1976).

When the cell membrane proteins of exponential and Cm incubated nucleoids were analyzed via gel electrophoresis, several MW

bands were obtained. Figure 8 revealed negligible differences in recoverable proteins isolated from cultures where protein synthesis was actively occurring compared to cultures where protein synthesis had been inhibited by the antibiotic. These results occurred as expected, since while Cm inhibited the synthesis of nascent proteins, the proteins synthesized during the exponential phase remained present in the lysate mixture.

Inner and outer membrane isolates from exponential and Cm treated cultures of RS46 were surveyed via gel electrophoresis to provide an index on the recoverable membrane-associated proteins. The profile depicted in Fig. 9 illustrated the membrane protein banding patterns of isolates from the isogenic F^- strain of E. coli. Numerous proteins were recovered from inner membrane samples of both exponential and Cm treated culture types. The array of recoverable proteins probably included the many functional proteins discussed by Davis et al. (1980) (i.e., transport, electron transport, cell wall biosynthesis and DNA replication). Outer membrane samples released many easily discernable protein bands, ranging in MW from approximately 14,000 to 66,000. A very prominent protein band was observed with an apparent MW of 24,000. Noticeable similarities were observed in the migrational patterns of several proteins from both culture types. These proteins possibly represented those associated with complexed regions of cytoplasmic and outer cell membranes, referred to as the zones of adhesion (Olsen et al., 1974; Portalier and Worcel, 1976; Gomez-Eichelman and Bastarrachea, 1975).

Electrophoresis of the inner cell membranes of the RS86 strain (Fig. 10) revealed protein banding patterns similar to those obtained from the F^- strain. Gel tracks containing the outer membrane proteins illustrated the recovery of 3 polypeptides with approximate molecular weights of 35,000, 44,000 and 46,000, increasing from bottom to top, respectively. These outer membrane proteins probably represented 3 major CM associated proteins, while the concentrations of proteins in the minor bands were not maximized enough by our lysis procedures to display sensitivity to the Commassie brilliant blue staining technique (as in the F^- strain). The isolation of the limited number of CM proteins may also be attributed to incomplete lysis of bacterial cells deep in the log phase and during Cm incubation such that some of these proteins adhered to the peptidoglycan layer which was removed during centrifugation.

Experimentation developed to analyze the proteins associated with freely sedimenting forms of ColE1-Ap plasmid DNA revealed noteworthy results. Data depicted in Fig. 11 illustrated the migration of 4 polypeptides into the 10% SDS polyacrylamide gel. The gel tracks of exponential, as well as Cm treated plasmid DNA revealed 3 high MW proteins, ranging from 40,000-48,000. One protein band was observed with an apparent MW of 36,000. Wolf-Watz and Norqvist (1979) were successful in isolating a 31,000 MW protein in association with purified chromosomal DNA via get electrophoresis. These investigators also suggested that this protein migrated to a region similar in MW to an CM associated protein.

The plasmid DNA purification technique utilized in our laboratory has not been functional in the yield of adequate amounts of plasmid DNA associated proteins for electrophoretic analysis. Additionally, dialysis performed on these samples to remove the high content of cesium chloride after centrifugation in the CsCl-EtBr density equilibrium gradient was inefficient and caused subsequent smearing of the sample in the gel track. Isolation of ColE1-Ap DNA associated proteins was acquired when the CLOS gradient technique was employed. Although these proteins have not been identified, they probably represented either: (1) The tandemly linked relaxation proteins of ColE1-Ap DNA; (2) cytoplasmic proteins which sedimented in the plasmid DNA region of the gradient; or (3) cell membrane polypeptides (which were fragmented from the nucleoid) involved in the complexing of ColE1-Ap DNA to the bacterial cell membrane. It is unlikely that the isolated proteins represented relaxation complex proteins since excised complexes would have migrated to a unit MW region in the gel. The recovered proteins probably did not represent cytoplasmic proteins since the centrifugation technique utilized differentially separated free macromolecular cell components. It is possible that one of the isolated proteins served to associate ColE1-Ap DNA to the bacterial cell membrane, while the other 3 existed in close proximity on the membrane and were not disturbed upon centrifugation.

Electrophoresis of freely sedimenting plasmid DNA associated proteins with concomitant electrophoresis of inner and outer

membrane proteins was depicted in Fig. 12. This data revealed observable similarities in some of the protein migrational patterns. Two polypeptides from plasmid DNA regions of CLOS gradients (both culture types) migrated to a MW region of about 48,000 and 36,000. Proteins of similar MW were apparent solely in gel tracks which contained inner membrane proteins. Proteins of similar MW have, however, been reported in association with outer cell membranes (Inouye, 1979). Two polypeptides observed (46,000 and 44,000 MW) in the plasmid DNA gel tracks were also observed in tracks which contained CM proteins. Minute traces of these proteins were noticed in the IM gel tracks.

A priori, we suggest that the proteins which associated with freely sedimenting forms of the plasmid DNA on CLOS gradients were involved in the anchorage of ColE1-Ap molecules to inner membrane fragments, possibly with contamination of outer membrane fragments. These proteins were, therefore, presumed components of the regions where the domains of cytoplasmic and outer bacterial cell membranes were associated. This presumption was based on the findings that similar MW proteins from IM and CM membrane isolates comigrated in separate gel tracks.

There is morphological evidence that the bacterial chromosome was attracted to adhesion zone sites frequently enough to exclude their adventitous association with the inner membranes at adhesion sites (Inouye, 1979). We further suggest that the association of ColE1-Ap DNA molecules to inner membrane fragments complexed with

outer membrane fragments at the zones of adhesion occurred for the purpose of replication and segregation. Association for the purpose of replication was highly suggested after gradient analysis of the in vivo localization of amplified ColEl-Ap DNA, which exemplified increased replicational rates (Clewell, 1972). Association for the purpose of segregation was inferred by the mini-cell data generated by Sparks and Helinski (1979). These findings described the isolation of ColEl-type DNA which associated with membranes in mini-cell preparations, free of chromosomal contaminations. It is our contention that amplified ColEl-Ap molecules accumulated on the cell membrane as a "bunch of grapes on a vine," awaiting partitioning of the bacterial cell for division. These events, in addition to chromosomal DNA replication were inhibited by Cm and, therefore, sequestered the plasmid DNA in a suspended state of anticipation.

CHAPTER VI

SUMMARY

The hypothesis that bacterial plasmid DNA attached to the bacterial cell membrane under conditions of Cm-induced plasmid amplification has been tested definitively. Observations revealed the following:

1. Intracellular localization of pulse-labeled ColE1-Ap after Cm amplification revealed a high level of membrane bound plasmid DNA on CsCl-sucrose gradients.
2. Vortexing of the plasmid DNA-membrane complexes prior to CsCl-sucrose centrifugation released appreciable amounts of the plasmid DNA from membrane templates. However, minute, recognizable amounts of plasmid DNA remained associated with membrane up to 4 hours after labeling.
3. Amplified ColE1-Ap DNA has been specifically recognized in association with the inner membrane fragments of the bacterial cell on isopycnic step neutral sucrose gradients.

4. Gel electrophoresis of proteins obtained from the freely sedimenting plasmid DNA regions of CLOS gradients revealed comigrational banding patterns with proteins isolated from the inner and outer cell membrane fractions of the isopycnic gradient.
5. The overlap in the molecular weights of the free plasmid associated proteins and the proteins isolated from purified IM and CM membranes is suggested to represent zones of adhesion which associate the two cell membranes.
6. That an aggregation of ColE1-Ap DNA on the cell membrane occurs as the "bunching of grapes on a vine". This type of aggregation is proposed to localize the plasmid DNA to ensure equal distribution into daughter cells, which never occurred in Cm treated cells.

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Doctor of Philosophy Thesis

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